1996

# Mitochondrial D-loop PCR-RFLP's Detect High Variability in a Paddlefish (*Polyodon spathula*) Population

Ronald L. Bromley
*Nebraska Game and Parks Commission Genetic and Forensic Laboratory*

# Mitochondrial D-loop PCR-RFLP's Detect High Variability in a Paddlefish (*Polyodon spathula*) Population

February 9, 1996

Ronald L. Bromley
Laboratory Scientist II
**Nebraska Game and Parks Commission**
**Genetic and Forensic Laboratory**
2200 N. 33rd St.
Lincoln, NE 68503

## Introduction

The paddlefish (*Polyodon spathula*) is an evolutionarily ancient, long-lived, highly migratory, cartilaginous fish native to the Missouri, Mississippi and several Gulf Coast drainages (Gengerke, 1986; Russell, 1986). Paddlefish populations have declined along with a concomitant reduction in their range partly due to impoundments, channel alterations and overharvesting. To address some of the problems that arise when restoration of a species such as paddlefish is undertaken, genetic analysis becomes necessary. To ensure that stocking programs are maximized, inbred and conversely, genetically unique fish populations must be identified. Previous genetic analysis was either biochemically performed or at the DNA-level. Livers were removed to isolate mitochondrial DNA which meant the sacrifice of the individual(s). ~~With the~~ Because of rapid advances in molecular biological techniques, non-invasive genetic analysis can now be obtained. This study demonstrates a method to analyze at the DNA level without the need to sacrifice the individual. By selectively targeting the genetic site of interest and using a technique known as PCR (polymerase chain reaction), millions of copies of a DNA fragment can be produced and then tested for variations between 2 or more individuals by restriction fragment length polymorphism (RFLP). ~~This allowed us to use small fin clips to evaluate the~~ genetic structure of a paddlefish population.

## Material and Methods

We obtained ~~fin clip~~ tissue samples (n=23) from paddlefish that were collected in the Gavins Pt. tailwater of the Missouri River. The tissue samples were placed in 1.5 ml microcentrifuge tubes and stored in liquid nitrogen for transport to the laboratory.

For this analysis, we examined the non-coding region found in mitochondrial DNA (mtDNA) known as the D-loop. This maternally inherited region is known to mutate 5-10 times faster than nuclear DNA and is suitable for phylogenetic analysis of closely related or recently diverged species.

*DNA Extractions*

Total DNA (nuclear and mitochondrial) was extracted from fin clips using a modification of the proteinase K-phenol/ chloroform method outlined in Ausubel, et al., 1994. After the DNA was extracted, the resultant DNA pellet was resuspended in TE (10 mM Tris, 1 mM EDTA, pH 7.4) and stored at -30°C until needed for PCR.

*Amplification, Restriction Enzyme Digestion, and Visualization of the PCR-RFLP's*

The mitochondrial D-loop was amplified using primers designed by Bernatchez et al., 1992 for a study of *Salmo trutta* and used for sturgeon as reported by Ferguson et al., 1993 are as follows: Phenyl-tRNA-1 (5'-GTGTTATGCTTTAGTTAAGC-3') and Proline-tRNA-2 (5'-ACCCTTAACTCCCAAAGC-3'). Amplification reactions were set up using 5 ul 10 x reaction buffer, 4 ul dNTP's, 0.5 ul of each primer, 1.0 ul of BSA (10 mg/ml), 8 ul of MgCl2 (10 mM), 28.5 ul of ddH20, 0.5 ul Taq polymerase. Amplifications were performed in a Perkin Elmer 480 thermocycler using 30 cycles of 94°C for 40 seconds, 55°C for 25 seconds and 72°C

for 120 seconds. Samples were denatured for 4 minutes at 95°C prior to amplification. After completion of PCR, all reactions were examined for quantity and size of amplicon by removing 1/6th of the reaction mixture and running it on a 0.8% agarose gel at 125 volts for 45 minutes with subsequent EtBr staining to visualize the DNA fragments. Negative controls (no DNA) were performed with each amplification to test for possible contamination. At no time did a negative control yield any detectable amplicon. All samples visualized by agarose gel electrophoresis demonstrated the sample size (~900 base pair) amplicons. After testing for correct amplification products, the rest of the reaction mixtures were subjected to restriction digestion using the following restriction enzymes: NlaIII, RsaI, Taq∝I, AluI and Tsp509I. The following restriction enzymes were found to not cut the amplicon or were non-informative (ie., monomorphic): HinPI, HpaII, Sau3AI and MseI. The restriction digests were performed per manufacturers protocol (New England BioLabs) and incubated between 4 to 16 hours. Following completion of the restriction digestion, the fragmented DNA was separated by polyacrylamide gel electrophoresis using a 6% polyacrylamide gel in a Hoefer gel electrophoresis apparatus. The gel was ran at 200 volts, for 6.0 hours. Following gel electrophoresis, the DNA fragments were visualized by silver staining using a modification of the protocol by Tegelstrom, 1994. Briefly, the gel was soaked in $ddH_2O$ for 20 minutes, then incubated in a silver nitrate solution (0.2 g silver nitrate in 125 ml of $ddH_2O$ to which 500 ul of 1 M NaOH and 500 ul of $NH_4OH$ was added). After the silver nitrate incubation, the solution was removed, the gel briefly rinsed in $ddH_2O$ for 10 seconds and a sodium carbonate solution (4 g of sodium carbonate in 200 ml $ddH_2O$ which 125 ul of formaldehyde was added). The gel was incubated in the sodium carbonate solution until the DNA bands were visualized (5-10 minutes). The reaction was stopped by removing the solution and soaking in $ddH_2O$ for at least 10 minutes. Gels were placed onto 3MM Whatman paper and vacuum dried using a Labconco Model 433-0100 gel dryer. DNA fragments were sized by use of a DNAStar digitizer and software.

**Results**

The haplotypes generated were assigned a letter based on the RFLP patterns generated, the most common RFLP pattern was given the letter "A", the next most common pattern "B", etc. The data and resultant haplotype frequencies are as follows:

| **Haplotype** | | **Frequency** |
|---|---|---|
| AAAAA | 6/23 | 0.2609 |
| AAAAB | 3/23 | 0.1304 |
| AAAAC | 1/23 | 0.0435 |
| AAAAD | 1/23 | 0.0435 |
| BBBBB | 4/23 | 0.1739 |
| BABBB | 1/23 | 0.0435 |
| BAABA | 2/23 | 0.0870 |
| BAABC | 1/23 | 0.0435 |
| BBBBA | 1/23 | 0.0435 |
| BAAAD | 1/23 | 0.0435 |
| CAAAE | 1/23 | 0.0435 |
| BAAAA | 1/23 | 0.0435 |

A haplotype diveristy was derived using the following formula: $h = n(1-\sum_i^2)n-1$ (Nei and Tajima, 1981). The diversity index was used to evaluate intrapopulational mtDNA lineages based on the haplotype frequencies found in the population.

| Haplotype | | Frequency |
|---|---|---|
| CAAA | 1/23 | 0.0435 |
| AAAA | 1/23 | 0.4783 |
| BAAA | 2/23 | 0.0870 |
| BAAB | 3/23 | 0.1304 |
| BABB | 1/23 | 0.0435 |
| BBBB | 5/23 | 0.2174 |

Diversity Index = 0.7271

| Haplotype | | Frequency |
|---|---|---|
| A | 10/23 | 0.4348 |
| B | 8/23 | 0.3478 |
| C | 2/23 | 0.0870 |
| D | 2/23 | 0.0870 |
| E | 1/23 | 0.0435 |

Diversity Index = 0.7035

| Haplotype | | Frequency | Haplotype | | Frequency |
|---|---|---|---|---|---|
| AAAAA | 6/23 | 0.2609 | BAABA | 2/23 | 0.0870 |
| AAAAB | 3/23 | 0.1304 | BAABC | 1/23 | 0.0435 |
| AAAAC | 1/23 | 0.0435 | BBBBA | 1/23 | 0.0435 |
| AAAAD | 1/23 | 0.0435 | BAAAD | 1/23 | 0.0435 |
| BBBBB | 4/23 | 0.1739 | CAAAE | 1/23 | 0.0435 |
| BABBB | 1/23 | 0.0435 | BAAAA | 1/23 | 0.0435 |

Diversity Index = 0.9011

When the first four restriction enzymes are used as an individual haplotype, the diversity index is 0.7271. Using the fifth restriction enzyme as its own haplotype, the diversity index is 0.7035. Combining the five restriction enzymes resulted in a diversity index of 0.9011.

**Discussion**

Previous genetic research on paddlefish has met with limited success. Protein electrophoresis has demonstrated limited variability (Carlson, et al., 1982; Epifanio, et al., 1989). Genetic analysis using total mitochondrial DNA RFLPs (Epifanio et al., 1989) demonstrated 6 haplotypes (or clones as they were referred to) throughout the paddlefish's native range. This research utilized a battery of six-base restriction endonucleases which limited the resolving power. Due to the development of PCR, it is no longer necessary to perform genetic analysis by isolating intact mitochondrial DNA. Specific DNA fragments can be selectively amplified and fragmented by four-base restriction endonucleases, thereby increasing the resolving power of genetic analysis. Furthermore, the organism being analyzed need not be sacrificed as only a small fin clip is necessary to acquire sufficient DNA.

To test whether increased resolution can be obtained through PCR-RFLP's, twenty-three samples were collected in the Gavins Pt. tailwater of the Missouri River. Polymerase chain reaction-restriction fragment length polymorphism targeting the D-loop found in mitochondrial DNA demonstrated 12 separate haplotypes. The restriction endonuclease Tsp509 I produced highly polymorphic profiles. Five unique variations were observed. When a diversity index was calculated for the population using only the Tsp509 I profiles, it compared favorably with the

diversity index of the variations observed from the first four restriction endonucleases (0.7035 vs. 0.7271, respectively). Both of these diversities are similar to known natural fish population diversities (Gyllensten and Wilson, 1987) of 0.72. But, if the profiles from all five digests were used, a rather large 0.9011 diversity index was calculated.

The large diversity index is likely an inflated value due to the fifth restriction site which may be lying in a hypervariable region of the D-loop. Similar highly polymorphic sites, near the $tRNA^{Phe}$ end of the control region, have been described in white sturgeon and humans (Brown et al., 1993). Therefore, the more probable diversity value lies somewhere between 0.70 and 0.72, which reflects a healthy natural population.

This study has demonstrated the ability to use PCR-RFLP's to genetically analyze a population without the need to sacrifice the study source which is very useful when working with threatened and endangered species. Our research has revealed a significant variability in one paddlefish population. It is now possible to continue the work necessary to characterize the paddlefish range easier, quicker and with more resolving power than previously used. In conjunction with the continuation of analyzing other populations using PCR-RFLP's directed towards the mitochondrial DNA control region, other sites such as the major histocompatibility complex (MHC) could be examined. This would allow for both the mitochondrial and nuclear DNA to be tested for genetic variability, thereby enhancing the understanding of the genetic makeup of paddlefish.

**Literature Cited**


Ausubel, F. M. (ed.). 1994. Current protocols in molecular biology. 3 vols. John Wiley & Sons, Inc. New York.

Bernatche, L., Guyomard, R., & Bonhomme, F. 1992. DNA Sequence variation of the mitochondrial control region among geographically and morphologically remote European brown trout Salmo trutta populations. Molecular Ecology. 1:161-173.

Brown, J. R., Beckenbach, A.T., and M.J. Smith. 1993. Intraspecific DNA sequence variation of the mitochondrial control region of white sturgeon *(Acipenser transmontanus)*. Mol. Biol. Evol. 10 (2):326-341.

Carlson, D.M., M.K. Kettler, S.E. Fisher, and G.S. Whitt. 1982. Low genetic variability in paddlefish populations. Copeia. 3:721-725.

Epifanio, J., Nedbal, M., and D.P. Phillip. 1989. A population genetic analysis of paddlefish *(Polyodon spathula)*. Aquatic Biology Technical Report 89/4. Illinois Natural History Survey. Pages 1-13.

Ferguson, M.M., Bernatchez, L., Gatt, M. Konkle, B.R., Lee, S., Malott, M.L., and McKinley, R.S. 1993. Distribution of mitochondrial DNA variation in lake sturgeon (Acipenser fulvescens) from the Moose River basin, Ontario, Canada. Journal of Fish Biology. 43 (Supplement A):91-101.

Gengerke, T.W. 1986. Distribution and abundance of paddlefish in the United States. Pages 22-35 in: J.G. Dillard, L,K. Graham and T.R. Russell (eds). The Paddlefish:Status, Management and Propagation. North Central Division, American Fisheries Society, Special Publication No. 7.

Gyllensten, U. and A.C. Wilson. 1987. Mitochondrial DNA of Salmonids: Inter- and Intraspecific Variability Detected with Restriction Enzymes. In: Population Genetics & Fishery Management. University of Washington Press. Seattle, WA., U.S.A. pp. 301-318.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Paabo, S., Villablanca, F.X., and Wilson, A.C. 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proc. Nat. Acad. Sci., USA. 86: 6196-6200.

Nei, M., and Tajima, F. 1981. DNA polymorphism detectable by restriction endonucleases. Genetics. 97:145-163

Russell, T.R. 1986. Biology and life history of the paddlefish--a review. Pages 2-20 in: J.G. Dillard, L,K. Graham and T.R. Russell (eds). The Paddlefish:Status, Management and Propagation. North Central Division, American Fisheries Society, Special Publication No. 7.

Tegelstrom, H. 1994. Detection of mitochondrial DNA fragments. In Molecular Genetic Analysis of Populations: A Practical Approach. A.R. Hoelzel (ed.). Oxford University Press, New York. 89-113.